

A Comparison Between Ascorbylpalmitateen Capsulated With Nanoliposomeas a Natural Antioxidant and Conventional Antioxidants (TBHQ and BHA) inthe Oxidative Stability of Sunflower Oil

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To improve the storage stability, sunflower oil containing poly unsaturated fatty acids (PUFAs) was encapsulated by using ascorbylpalmitate encapsulated with nanoliposomes (APEWN). Ascorbylpalmitate was selected as the wall material because it showed a better antioxidant activity. Microcapsules were prepared and compared with common antioxidants such as BHT and TBHQ. Rancimat method was used for the initial screening of the antioxidant activity. Free fatty acids (FFA), peroxide value (PV), anisidine value and Iodine value of the samples were monitored. The Results showed micro encapsulation of ascorbylpalmitate was suitable for preventing sunflower oil from oxidizing in comparison with TBHQ and BHT.

Key words: lipid oxidation, ascorbylpalmitate, nano- liposome, TBHQ, BHT, sunflower oil.

Sunflower oil is a high-quality edible oil. It is used in cooking, frying, and in the manufacture of margarine and shortening. Sunflower oil is widely used in nutrition as a source of high linoleic acid and vitamin E, compared to other vegetable oils. Furthermore, it is light in taste and appearance. The health benefits associated with polyunsaturated fatty acids (PUFAs) include the anti-inflammatory properties and their positive effects on blood pressure, heart and nervous system function. Sunflower oil has a large content of polyunsaturated fatty acids (Shahidi *et al.* 1996).

Oil oxidation is an undesirable series of chemical reactions involving oxygen that degrades the quality of oil. The unsaturated long-chain fatty acids present in fats and oils, readily oxidize when exposed to heat, light, and air (Sikwese and Duodu 2007).

It is well known that edible oils are used as cooking media at high temperatures in the presence of oxygen. Therefore, they are subjected to thermo-oxidation, polymerization, and hydrolysis which eventually cause rancidity in oil, accompanied by off flavor and off-odor, and consequently a decrease in the nutritional quality of the product (Siddhuraju and Beeker 2003; Min and Lee 1998).

The oil is usually exposed to oxidation which cannot be stopped completely, but there are

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ways to reduce it. Therefore, attempts should be made to reduce oxidation at each stage of oil manufacture. Different strategies have been used to protect PUFAs from oxidation. A common method of reducing oxidation is the use of a natural (Huber, *et al.* 2009) or synthetic antioxidant.

Chemically synthesized compounds such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are used as antioxidants in oil products. The use of BHA and BHT has proved to have toxic and carcinogenic effects (Krishnaiah *et al.* 2010). Therefore, there is an increasing interest in the antioxidant activity of natural compounds. Phospholipids have demonstrated antioxidant activity (Khan and Shahidi 2000), and their synergistic effects have been observed in the protection of oil.

Encapsulation is another approach to protecting PUFAs. The encapsulation of PUFA-containing oils increases their oxidative stability significantly and reduces the undesirable odors of volatile oxidation products. The use of liposomes may be a suitable and promising method to increase the bioavailability and stability of PUFAs and to overcome their drawbacks (Rasti *et al.* 2012).

The ability of liposomes, the multiple or single phospholipid bilayers surrounding an aqueous medium, as a biocompatible delivery system to help overcome the problem of the reduced solubility of many herbal extracts in lipids is very important (Mozafari *et al.* 2008; Maherani *et al.* 2012). Liposomes are an important area in nutritional, biological, and pharmaceutical research, as they are among the most effective carriers for the introduction of various types of bioactive agents into target cells and different parts of non-living systems such as food products (Gregoriadis 2007; Mozafari *et al.* 2008).

Ascorbyl palmitate is a fat-soluble form of ascorbic acid. This makes ascorbyl palmitate a very attractive form of vitamin C supplementation. Ascorbyl palmitate can be stored in cell membranes until it is required by the body. A major role of vitamin C is in the production of collagen, a protein that forms the basis of connective tissue - the most abundant tissue in the body (Reynolds 1996). Ascorbyl palmitate is an effective free radical-scavenging antioxidant which

promotes skin health and vitality. Ascorbyl palmitate, influencing the cell membrane, provides the antioxidant potential comparable to or even greater than that of vitamin E (Kristl *et al.* 2003). It also acts synergistically with vitamin E, helping regenerate vitamin E radical in a basic form. For an improved immune system response and advanced antioxidant properties, the advantages of the full health benefits offered by ascorbyl palmitate should be taken (Cort, 1974). McMullen *et al.* (1991) suggested that the antioxidant effects of ascorbic acid could be more apparent in an oil system if ascorbic acid was used in combination with other antioxidants such as tocopherols, BHT, and BHA. In fat systems, the use of fat soluble esters of ascorbic acid such as ascorbyl stearate or ascorbyl palmitate is recommended.

In recent years, liposomes have been extensively studied as carrier systems for medical (Khosravi-Darani and Mozafari 2010; Khosravi-Darani *et al.* 2010; Khosravi-Darani *et al.* 2007; Mortazavi *et al.* 2007; Mozafari and Khosravi-Darani 2007) and food (Vafabakhsh *et al.* 2013; Mozafari *et al.* 2008; Jahadi *et al.* 2012; 2015; Ebrahimi Khoosfi *et al.* 2014; Khosravi-Darani *et al.* 2016; Khanniri *et al.* 2016) purposes; however, only a few works have been published dealing with the antioxidants encapsulated with nano-liposomes in vegetable oils.

The aim of this work was to evaluate the antioxidant activity of ascorbyl palmitate and to increase the effectiveness and stability of ascorbyl palmitate, as well as control its delivery during storage by nano-encapsulation with nano-liposomes, and to compare them with the known synthetic antioxidants, namely BHT and TBHQ.

MATERIALS AND METHODS

Materials

Sunflower oil without antioxidants (neutralized, bleached and deodorized) was supplied from Nina, Iran. BHT and BHA were purchased from Sigma Chemical Co. Eicosapentaenoic acid (EPA) and Docosahexanoic acid (DHA) from Novelty, Puchong Co Malaysia, soybean phospholipids from the Andre Imcopa, Santo Co Brazil, Tetraethoxypropane 1/1/3/3 and

Thiobarbituric acid (TBA) from Sigma-Aldrich, St. Louis, Mo, USA were purchased. Other chemicals, including methanol, chloroform, n-hexane, ethanol, ferric chloride, and glycine were provided from Merck, Germany.

All tests were carried out at two temperatures of 25°C and 180 °C. The samples of oil were formulated at 180 °C for 24 hours, heated and then prepared at 6 h intervals and packed under the nitrogen condition at -18 °C until experiments and the oil stability index was measured again.

Methods

Liposome preparation

Empty and encapsulated-PUFA liposomes were prepared according to the procedure previously described by Colas *et al.* (2007). Briefly, the mixture of the liposomal ingredients, including soybean phospholipids (PLs) (preheated to 30°C) and PUFAs (DHA and EPA; 2:3, w/w) with a mass ratio of 2:0.4, were hydrated by adding deionized water and glycerol (final concentration 2% v/v). The mixture was preheated to 30°C and stirred at 1000 rpm using a hotplate stirrer (MAXIMA Digital, Fisher Scientific, Shah Alam, Malaysia) on a hotplate (IKA_C-MAG HS 10, Petaling Jaya, Malaysia) at 30°C for 60 min. The preparation process was carried out in a six-baffled glass vessel. Compared to the conventional method, in this method, liposomes are prepared by direct hydration and without solving the PLs and FAs in organic solvents. In order to anneal and stabilize the liposomal samples, they were kept at 25°C (ambient temperature) under nitrogen for at least 1 h after preparation.

APEWN, BHT, and TBHQ as the most powerful antioxidants were added to sunflower oil at different concentrations (rapid oxidation of this oil was the reason for choosing it). The formulated-with-antioxidant and control samples were studied at 25 °C and 180° C during 45 days of storage on the days 0, 15, 30 and 45.

Oil stability

Measurement of the Peroxide value

Primary oxidation products, hydroperoxides, were determined by the peroxide value measurements. 1_0.1 g of the oil was weighed and subjected to the iodometric determination of the peroxide value (AOCS 1999).

The induction period was considered as the number of days needed for the peroxide value

of the sample to reach 20 meq O₂/kg of the edible oil (Economou *et al.* 1991).

Measurement of the Iodine value

The oil samples, weighing 0.25 g, were poured into a completely dry 500-ml flask. Then, each sample was dissolved in 10 ml of chloroform and 25 ml of the Hanus reagent was added to the solution with a bubble pipette. The Erlenmeyer flasks were capped, kept in the dark, and shaken every once in a while. After 30 minutes, 20 ml of 15 % potassium iodide solution was added to each Erlenmeyer flask and the flasks were vigorously shaken. 100 ml of freshly boiled and cooled distilled water were poured into each flask, taking care that the cap and the inside walls of the flasks were also washed so that any excess iodide, adhering to them, was also washed into the solutions inside the flasks. The solutions were then titrated by using 0.1 N sodium thiosulfate. The contents of each flask were repeatedly stirred and the titration went on until the solution turned yellow. At this stage, 2 – 3 drops of the starch reagent were added and the titration continued till the blue color completely disappeared. Care was taken to vigorously shake the flasks near the end of the titration so that any iodine particles remaining in chloroform were absorbed by the potassium iodide solution. Together with the oil samples, the test was also performed on the control. The iodine value was calculated using equation 1:

$$IV = \frac{12.69C(V_1 - V_2)}{M} \quad \dots(1)$$

where N is the normality of the sodium thiosulfate solution, B denotes the ml of the thiosulfate used for the control sample, S represents the ml of the thiosulfate used for the samples, W stands for the weight of the sample (g), and 126.9 is the molecular weight of iodine (AOAC 2005).

Measurement of the Anisidine value

p-Anisidine was dissolved in glacial acetic acid to make up a 0.25g/100ml solution. Isooctane was used as a solvent for the oil samples. The test was conducted in triplicate for all samples. 0.5-0.7g of the oil samples was accurately weighed in 25-ml volumetric flasks, noting down the masses of the samples used. They were then diluted to the volume with isooctane. The absorbance values (Ab) of the resulting solutions were measured at

350 nm using isooctane as blank. Glass cuvettes were used for all absorbance measurements. 5ml of the solution was then transferred to a test tube and 5ml of isooctane to another test tube. 1ml of the p-anisidine solution was added to both of them and the solutions were mixed. After 10 minutes, the absorbance values (Ab) of the sample solutions were read with isooctane as blank. The p-Anisidine values were calculated using the following equation (Eq.2): (AOAC 2005).

$$q. (2): P - A.V = \frac{25 \times (1.2As - Ab)}{m} \quad \dots(2)$$

Measurement of the Acid value

Each oil sample (1.0 g) was weighed and dissolved in 50 ml of ethanol in a conical flask. Two drops of phenolphthalein were added and titrated to pink end point (which persisted for 15 minutes) with 0.1 N potassium hydroxide (KOH). Acid value was calculated as follows:

$$\text{Acid value} = 56.1 V C m$$

where 56.1 is the equivalent weight of KOH, V is the volume (ml) of the standard volumetric KOH solution used, C is the exact concentration of the KOH solution used (0.1 N); m is the mass (g) of the test portion (1 g). (AOAC 2005).

Evaluation of the antioxidant activity by the Rancimat method

The method used, was adapted from Gortziet *al.* (2006). The antioxidants namely, BHT, TBHQ and APEWN all at 150 ppm -were accurately added to stripped sunflower oil (Elais S.A., Athens, Greece) and the samples prepared using the method described by Fuster *et al.*, (1998) and their effects were determined using a Rancimat 679 (Metrohm LTD, Herisau, CH 9101, Switzerland), along with another sample of sunflower oil without antioxidant (control). 1 mL of the appropriate solvent (MeOH or CH₂Cl₂) was added in order to dissolve the antioxidant. The conditions were set at 90 °C and 15 L/h.

The protection factor (P.F.) was calculated as:

$$P.F. = (\text{induction period with antioxidant}) / (\text{induction period without antioxidant}).$$

A protection factor greater than 1 indicates the inhibition of the lipid oxidation. The higher value represents the better antioxidant activity.

Statistical analysis

The data analysis of variance (ANOVA) was performed using SPSS version 17. Duncan's multiple range test was carried out for mean comparison at the 95% significance level (p<0.05). Each treatment was repeated in triplicate.

RESULTS AND DISCUSSION

Peroxide values (PV)

PV is widely used as a measure of the primary lipid oxidation, indicating the amount of peroxides formed in fats and oils during oxidation (Ozkan *et al.* 2007). The effects of BHA, BHT and APEWN added to the sunflower oil samples, are shown in fig 1. It was regular increase in PV for all the samples over the storage period. This rise can be attributed to the formation of hydro-peroxides as the initial products of the oxidation of the oil samples. The total increment in PV is as follows in descending order: SFO (control) > APEWN > BHT > TBHQ (Fig.1). There were no significant differences between the peroxide values of the oil samples formulated with APEWN and BHT during 15 days of storage, but TBHQ had a lower peroxide value during this period. However, when the sunflower oil samples were stored for 30 to 45 days, APEWN was more effective in controlling the peroxide value, thus the oxidation of the sunflower oil samples was delayed and the antioxidant activity was enhanced with APEWN during storage.

The results of the peroxide value measurements at 180 °C showed that the total PV was increased as follows: SFO control > APEWN > BHT > TBHQ (Fig.2). There were no significant differences between the peroxide values of the oil samples containing BHT and TBHQ and APEWN at 180 °C during storage. The addition of APEWN brought about a more intense antioxidant activity (significant at P<0.05). This means that APEWN, as a natural antioxidant, can substitute for synthetic antioxidants in reducing the peroxide values of the sunflower oil samples.

In that phase, the formation of hydroperoxides was shown by their decomposition into secondary products. Yanishlieva *et al.* (1996) declared that ascorbyl palmitate was a better antioxidant in oils. Liolios *et al.* (2009) showed that the carvacrol encapsulated with nanoliposome presented a better antioxidant effect compared with the pure samples. Satyanarayana *et al.* (2000) stated

that ascorbylpalmitate helped control the formation of hydroperoxides in frying oils in comparison with other antioxidants, including BHA, BHT, PG. They also cited that the addition of ascorbylpalmitate to frying oil reduced the PV of the extracted fat during storage. Vayupharp and Laksanalamai (2012) claimed that the incorporation of grape seed into fried pork inhibited or delayed rancidity, and also there was no statistically significant difference in the rancidity as shown by the PV values of the fried pork with BHT and grape seeds.

Iodine value

The iodine value is a measure of the unsaturation of oils. It is one of the parameters used to measure the oil quality (Rehab 2012). Fig 3 demonstrates the IV of the sunflower oil formulated with various types of antioxidants at 25 °C. Sunflower oil with various levels of an antioxidant during storage at 25 °C experienced a significant ($P < 0.05$) decrease in iodine values (degree of oil unsaturation). This decrease was due to the increase in the predominance of monounsaturated fatty acids in the oil samples. The initial iodine values of the control were 131.99 to 60 g, but 132.01 to 92 g in sunflower oil with antioxidant. The antioxidant reduced the oxidation rate of PUFAs, as detected by the relatively low reduction in the iodine values. There were no significant differences between the iodine values of the oil samples formulated with APEWN and BHT and TBHQ during storage at 25 °C (Fig.3).

The IV of sunflower oil with different antioxidants during deep fat frying at $180^{\circ}\text{C} \pm 5^{\circ}\text{C}$ are depicted in Fig.4. The frying process induced a significant ($P < 0.05$) decrease in the IV of all oil samples under study. It is well known that during frying, some of the non-conjugated double bonds are converted to conjugated ones. The conjugated system, in general, precludes the complete addition of iodine (Rehab 2012). This fact indicates the decrease of IV for the samples under study during frying at $180^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 45 days of storage. The decrease in iodine value denotes the decrease in the degree of unsaturation of the oil caused by the extent of oxidation (Kirk and Sawyer 1991; Rehab 2012). The highest decrease in IV was recorded for the sunflower oil without antioxidant due to the reduction percentage in the iodine value which ranged from 118.03 to 75 at the end of the frying period. On the other hand, the samples

with APEWN, BHT and TBHQ had significantly the smallest reduction in their iodine values which were 95, 92.06 and 96 at the end of the frying period, respectively. Autoxidation of the samples affected their fatty acid composition, as polyunsaturated fatty acids were oxidized faster than the saturated and mono-unsaturated ones (Semwal *et al.* 1996). The addition of antioxidant to sunflower oil during frying effectively reduced the oxidation rate in sunflower oil, as detected by the relatively low reduction in the iodine values (fig 4). The results showed that APEWN was not significantly different from BHT and TBHQ which can replace them as a natural antioxidant.

Anisidine value

During lipid oxidation, hydroperoxides, the primary reaction products, decompose to produce secondary oxidation products (aliphatic aldehydes, ketones, alcohols, acids, and hydrocarbons) which are more stable during the heating process, responsible for off-flavors and off-odors of edible oils (Poiana 2012). Sunflower oil had the highest *p*-anisidine values, because of oleic acid and linoleic acid, the main types of unsaturated fatty acids that were oxidized, present in the highest amount of sunflower oil (88%) (Allen and Hamilton 1989).

The control sample (without any extract) had the highest *p*-anisidine value and thus indicated a higher rate of oxidation. Among different methanolic and acetone extracts, 80% methanolic (Table 1) presented the changes in *p*-AV at 25 °C and 180 °C as affected by supplementation with APEWN, BHT, and TBHQ. The control sample (without any antioxidant) had the highest *p*-anisidine value and thus indicated a higher rate of oxidation.

The addition of APEWN, BHT, and TBHQ resulted in a significant decrease in *p*-AV ($p < 0.05$). The result of *p*-AV at 25 °C and 180 °C showed that the total *p*-AV was increased as follows: SFO control > BHT e" APEWN e" TBHQ. TBHQ provided the best protection against the secondary oxidation of the oil samples. However, SFO treatments with APEWN showed reduced *p*-AVs compared with those of BHT.

Poiana (2012) reported that grape seed extract (GSE) provided the best protection against the secondary oxidation of the sunflower oil samples in comparison with BHT. They attributed

these results to the polyphenolic compounds found in GSE that had a strong inhibitory effect on the secondary lipid oxidation.

Acid value

The FFA contents of SFO (control), SFO-TBHQ, SFO – BHT and SFO – APEWN during 45 days of accelerated storage at 25°C and 180°C are shown in Table 2. When comparing among different samples, the total increment of the FFA content during 45 days of storage was in the order of SFO (Control) > SFO – BHT > and SFO – APEWN > SFO-TBHQ. APEWN exhibited excellent antioxidant activity in comparison with BHT. The FFA of SFO from day 0 till day 45 and at the frying temperature with APEWN controlled the FFA oxidation well throughout the storage period.

The antioxidant activity

The fat and oil oxidation stability is commonly assessed by the fully automated version of active oxygen method available in Rancimat apparatus and is accepted as a standard method by American Oil Chemists' Society (AOCS (Cd 12b-92) 1999) (Hamed *et al.* 2012). Rancimat method determines the induction period by measuring the increase in volatile acidic by-products released from the oxidizing fat at 100- 110 °C. The concentration of degradation products which are transferred to distilled water is monitored by measuring conductivity. Longer induction periods suggest the stronger activity of the added antioxidants (Hamed *et al.* 2012).

The control sample (sunflower oil without any antioxidant) showed the shortest induction period (3.28 hr). The descending order of antioxidant capacity was TBHQ > APEWN > SFO – BHT > control (Fig.5). Hamed *et al.* (2012) suggested that TBHQ revealed the highest protection as indicated by its longest induction period (12.3 hr).

Špiclin *et al.* (2001) expressed the stability of ascorbylpalmitate is highly dependent on its initial concentration, its location in the microemulsion, the amount of oxygen dissolved in the system and the storage conditions. Possible approaches to increase its stability include the proper selection of the type of microemulsion and the appropriate choice of initial concentration and storage conditions. Other factors also have to be considered in formulating an optimal microemulsion as a carrier for ascorbylpalmitate, for example, the selection of non-oxidizable components of the

carrier system. Nevertheless, ascorbylpalmitate is still convenient as an antioxidant to stabilize formulations.

The modified antioxidant action of the extract resulting from its encapsulation was expected, since the complex (liposome membrane-fraction) possesses new physicochemical characteristics and bioactivity dependant on structure, size and ζ -potential of the preparation. Liposomes containing the extracts were more stable than those extract-free. This may imply that lipophilic substances used in the extracts and phospholipids were packed regularly and tightly, conferring the membrane rigidity and decreasing the membrane permeability (Gortzi, *et al.* 2007). Jurkovic *et al.* (2003) applied ascorbylpalmitate in microemulsions for scavenging free radicals formed in UV irradiated porcine skin. ascorbylpalmitate acts as an antioxidant. Due to its chemical structure and amphiphilic nature, the molecules of ascorbylpalmitate are orientated in the lipid bilayers with the palmitic residue in the lipophilic phase and the lactone ring in the lipid-water interphase. They suggested the effectiveness of ascorbylpalmitate was dependent on its concentration and the type of microemulsion. Hrašić *et al.* (2000) showed the higher antioxidant activity of ascorbylpalmitate in sunflower oil compared with β -tocopherol and citric acid.

CONCLUSIONS

The present study has demonstrated the potential antioxidant preservability of ascorbylpalmitate. Encapsulation in liposomes modified the activities of ascorbylpalmitate. Ascorbylpalmitate was compared with BHT and TBHQ, BHT and TBHQ are synthetic antioxidant products, widely used in oil. Our results suggested that APEWN would be an effective natural antioxidant in sunflower oil. These results also suggested that ascorbylpalmitate has the potential to be used as a naturally derived antioxidant for specialized food application in processed food products.

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